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14. ABSTRACT
Telomerase, a ribonucleoprotein enzyme composed of an RNA template (hTR) and a catalytically active protein subunit (hTERT), synthesizes telomeres after cell divisions and is obligatory for continuous tumor cell proliferation as well as malignant progression of breast cancer cells. Telomerase is an attractive anti-cancer therapeutic agent because telomerase activity is present in over 90% of human breast cancers but is undetectable in most normal somatic cells. Traditional therapies (surgery, chemotherapy, radiotherapy, etc.) lack the ability to effectively control and cure breast cancer, primarily because residual cells are or become resistant to DNA damaging modalities including standard chemo- and radio-therapies. Since telomerase requires its associated hTR for repeat synthesis, we have chosen to use RNA interference as a method to inactivate hTR and hence telomerase. RNA interference (RNAi) has become a powerful tool for the analysis of gene function in that RNAi allows sequence specific inhibition of gene expression. Another protein we targeted is p21, which has long been established as a requirement for senescence. We wanted to further examine its relationship to senescence and apotosis, in an attempt to sensitize breast tumor cells more effectively.

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Introduction

Last year over 203,500 new cases of invasive breast cancer occurred among women in the United States (American Cancer Society, 2002). Currently, the primary treatment for breast cancer consists of surgery and adjuvant therapies including chemotherapy, hormone therapy, and localized radiation. Despite the initial success of these clinical approaches, the frequent recurrence of breast cancer indicates that resistance to therapy is common in breast tumors. Associated with over 90% of malignant breast cancer, telomerase is a reverse transcriptase containing a catalytic protein component, hTERT, and an RNA template, hTR, for catalyzing the addition of telomeric (TTAGGG) DNA repeats onto the chromosome ends (8,18). As such a prominent molecular marker for human cancer, telomerase has proven useful for detection of recurrent disease, as well as a promising target for adjuvant cancer therapy, especially for breast cancer treatment (15). Traditional therapies (surgery, chemotherapy, radiotherapy, etc.) lack the ability to effectively control and cure breast cancer, primarily because residual cells are or become resistant to DNA damaging modalities including standard chemo- and radiotherapies. Since telomerase requires its associated hTR for repeat synthesis, we have chosen to use RNA interference as a method to inactivate hTR and hence telomerase. RNA interference (RNAi) has become a powerful tool for the analysis of gene function in that RNAi allows sequence specific inhibition of gene expression (1,4,9-14).

We chose to study the RNAi mechanism in human breast cancer cells because the process is not yet fully understood and because of its potential as an attractive anti-telomerase breast cancer option. The siRNAs we will use are directed at the hTR portion of telomerase, which is a modification of the traditional RNAi in that hTR is a functional RNA and not an mRNA. The focus of this proposal is to examine the mechanism of RNAi after knockdown of hTR, define the mechanism of silencing for each targeted sequence, and determine if this inhibition sensitizes breast cancer cells to standard breast cancer therapies. The goal is to extend upon our exciting preliminary results to better understand the mechanisms of RNAi and the effects of telomerase inhibition on mainstay treatments of breast cancer cells. Another protein we targeted is the cyclin-dependent kinase inhibitor, $p21^{waf-1}$, which has long been established as a requirement for the onset of cellular senescence. We wanted to further examine its relationship to senescence and apoptosis after treatment of breast tumor cells, in an attempt to sensitize these cells more effectively in hopes to reduce collateral effects on surrounding normal cells.

Body

To date, we have utilized the established MCF-7 breast cancer to study the effects of the targeted siRNAs. Initially, we created two siRNAs that target different portions of the functional hTR RNA. The first position is located in the single stranded template region, which will be called hTR-T, and the other is complimentary to the conserved pseudoknot domain (CR2/CR3) domain, which will be called hTR-2 (Figure 1 & Table 1) (3).

As a first step, synthetic siRNAs were created that are 21 nucleotides in length paired in a manner to have 2 nucleotide 3' overhangs on each end. Each siRNA was transiently transfected into MCF-7 cells in two separate experiments. We observed an approximately 80% reduction in telomerase activity over a 72-hour time period in the hTR-2 treated cells and a slightly smaller reduction in the hTR-T treated cells (Figure 2 & data not shown). This provides the necessary proof of principle experiment demonstrating that the vector-mediated expression of

these siRNAs will inhibit telomerase. In addition, the preliminary experiments are noteworthy in that RNAi is normally regarded as limited to the cytoplasm, but because telomerase is predominantly nuclear, it is quite possible that the knockdown of telomerase transpired in the nucleus.

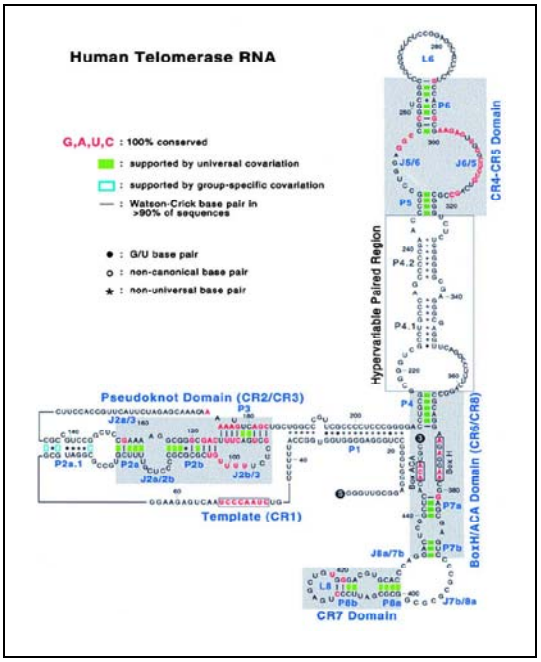


Figure 1. Proposed Secondary Structure of Human Telomerase RNA. This schematic displays the proposed L-shape secondary structure of the functional hTR RNA. There are ten conserved helical regions (P2a-P8b), which constitute the four universal structural domains known as the pseudoknot domain, the CR4-CR5 domain, the Box H/ACA and the CR7 domain. These are all shaded in gray and labeled. Note the single-stranded and labeled template region, which we targeted with hTR-T (taken from Chen et al. 2000 (3)).

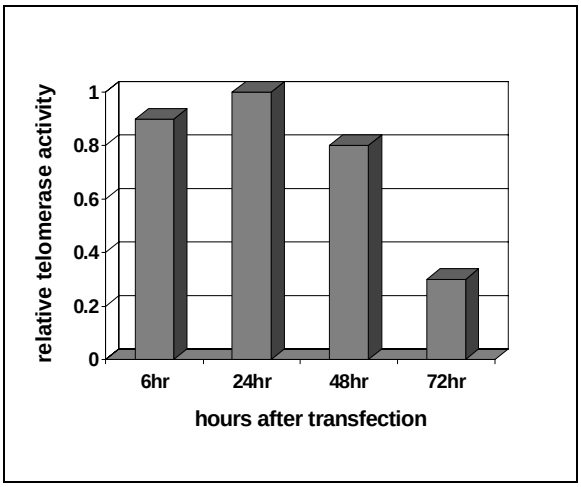


Figure 2. Decline in telomerase activity using siRNA targeted against hTR. siRNA was designed and directed against a critical pseudoknot (hTR-2) found in the predicted secondary structure of the telomerase RNA template, hTR. After transfection, cells were harvested and tested for telomerase activity using the telomere repeat amplification protocol (TRAP) at the indicated times. The relative telomerase activity was calculated as a percentage of the 24-hour time point. We observe a reproducible 3 to 4-fold decline in activity after 3 days of treatment.

In order to determine if telomerase could be knocked down further and for a greater length of time, we chose to stably express these sequences into the MCF-7 cells. The pSUPER-retro vector, which is specifically designed for RNAi, enabled us to accomplish this task using retroviral infection and selection with puromycin for stable integration of the vector sequences. After transcription/expression of the siRNA, the RNA sequence will fold into a 64 base pair hairpin loop that will be cut *in vivo* by a Ribonuclease III enzyme called dicer, creating the same length 21bp siRNAs as the synthetic version (2). Thus far, the hTR-T and hTR-2 sequences have

been cloned into the vector and have been successfully infected into MCF-7 cells followed by cylinder cloning.

Table 1. Description of Telomerase-Targeted siRNAs

siRNA Name	hTR Target Sequence (5'-3')	Conserved Target Region
hTR-T	48-AACCCUAACUGAGAAGGGC-66	Template (CR1)
hTR-2	175-AAUGUCAGCUGCUGGCCCG-193	Pseudoknot Domain (CR2/CR3)- P3
hTR-3	302-GAGUUGGGCUCUGUCAGCC-320	CR4-CR5 Domain
hTR-4	373-GAGGAACGGAGCGAGUCCC-391	BoxH/ACA Domain (CR6/CR8)
hTR-5	405-AUUCCCUGAGCUGUGGGAC-413	CR7 Domain

***Objective #1*: Define the mechanism of telomerase inhibition using targeted short interfering RNAs (siRNAs) in breast tumor cells.**

The minimal catalytic core components of human telomerase consists of a protein subunit (hTERT) and an RNA component (hTR) that serves as a template for synthesis of telomere repeats. The siRNAs we have created target two different portions of the functional hTR RNA. We will ascertain by what method the siRNAs block the function of hTR and telomerase activity. In 1998, Sharp and McManus (14) claimed that the siRNAs form a silencing complex that causes a sequence specific mRNA degradation, but we want to test this hypothesis for the structural and functional hTR RNA, as well as several other mechanisms. Specifically, one plausible theory is that perhaps the siRNAs simply obstruct the functional activity of hTR without degradation as more of a microRNA function rather than RNAi (14). The siRNAs association with hTR may disrupt secondary structure, or block hTR and hTERT assembly, thereby terminating telomerase's functional activity. Another possibility pertains to the actual association of telomerase and telomeres, where the siRNA may interrupt this entirely and lead to telomere shortening, which will be difficult to investigate given the limiting reagents currently for detection of telomerase (hTERT) at the telomere.

Determining if Targeted siRNAs Cause Degradation:

In two separate *in vitro* assays, we examined the levels of hTR using RT-PCR, after exposure to the targeted synthetic siRNAs. In the first instance hTR was transcribed from the T7 promoter (pGEM5Z) and followed by incubation with various concentrations of the siRNAs. In the second assay, we used PCR generated hTR RNAs, one full length and the other missing the first 32 base pairs, in case the upstream 90 base pair sequence from the vector interfered with the siRNAs' ability to cause degradation. In both experiments no changes in the constitutive levels of hTR RNA were observed (Figure 3).

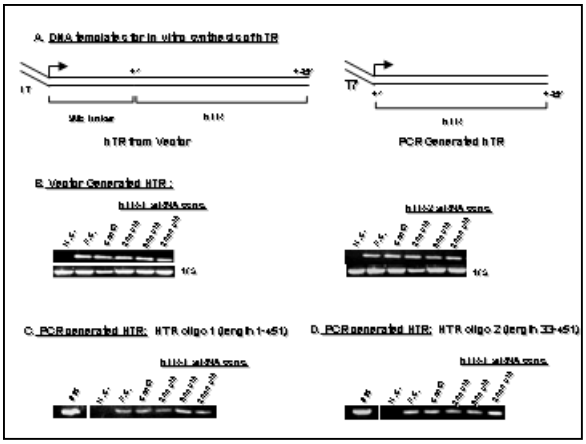


Figure 3. Determining degradation *in vitro* using RT-PCR. Levels of hTR were measured after incubation with increasing concentrations of the synthetic siRNAs hTR-T and hTR-2. The telomerase template RNA, hTR, was transcribed from two different sources, vector and PCR generated (A). In both instances there were no alterations in the amount of hTR indicating that degradation did not occur ***in vitro*** (B&C).

Examining hTR Secondary Structure Without Degradation: Through the use of native gel electrophoresis, we examined the effects of siRNA on hTR folding *in vitro*. hTR was transcribed from T7 promoter (pGEM5Z) and in the presence of ^{32}P-UTP, followed by the addition of the siRNAs in increasing concentrations and electrophoresis on a non-denaturing polyacrylamide gel. We did not observed changes in conformation by comparing those samples containing the siRNAs against those that are lacking the inhibitors (Figure 4). We saw no evidence that the hTR siRNAs disrupt secondary structure because there were no distinct differences in hTR migration in terms of folded versus unfolded RNA. Our next step is to express hTR in the presence of the siRNAs (i.e. during transcription) followed by native gel electrophoresis, which will verify that the siRNAs block folding in case already synthesized (and folded) hTR cannot be altered.

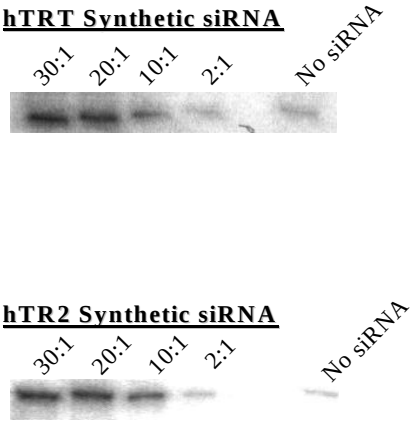


Figure 4. *In vitro* comparison of migration of hTR with and without the siRNAs. hTR RNA was radiolabeled with ^{32}P and incubated with various concentrations of the synthetic siRNAs hTR-T (upper panel) and hTR-2 (lower panel). Changes were not observed in either the migration or conformation of hTR on a native gel, suggesting that the synthetic siRNAs did not modify the secondary structure of hTR.

Investigating hTR and hTERT Complex Formation: The interaction of the two core components of telomerase will be explored *in vitro*, by two independent methods involving immunoprecipitating hTERT protein to look for an association with hTR RNA and then by precipitating hTR to look for an association with hTERT protein. Initially, hTR will be *in vitro* transcribed in the presence of ^{32}P-UTP. The siRNAs will then be added to the radioactive hTR

or will be included in the hTR synthesis, with a no siRNA negative control. In a separate system, the hTERT will be synthesized *in vitro* using a TnT reaction (Promega) and labeled with ^{35}S-methionine, and the hTR and hTERT mixtures will be combined/assembled. We will then immunoprecipitate with hTERT antibodies (Calbiochem, San Diego, CA or Alpha Diagnostic, San Antonio, TX) in the presence and absence of the siRNAs or IgG as a negative control and determine if radiolabeled hTR is precipitated. Using an 8M urea gel electophoresis, hTR will be detected using the Phosphoimager (Molecular Dynamics).

The second experiment will involve RNA retrieval using a biotinylated hTR and ^{35}S-hTERT with and without the siRNAs as we have previously done (17). hTR and hTERT will be assembled *in vitro* at 30°C for two hours. hTR will be retrieved using streptavidin-coated paramagnetic beads to bind the biotin-labeled hTR complexes. Beads will be captured with a heavy magnet, and radiolabeled hTERT will be detected following SDS-PAGE (8%). From this, we will be able to semi-quantitatively evaluate the amount of hTERT precipitated in the presence and absence of the siRNAs in order to determine if the interaction is altered by incubation with the siRNAs.

Telomerase and Telomere Interaction: Detecting the association of telomerase with telomeres will be accomplished using chromatin immunoprecipitation (ChIP) assay (15). In this procedure, cells will be fixed with formaldehyde, which serves to cross-link proteins bound to DNA. DNA will then be isolated, sheared, and immunoprecipitated with an antibody to the hTERT protein. After reversing the crosslinking, DNA will be blotted onto nylon and hybridized with a radiolabeled telomeric probe. We hypothesize that with one or more of these siRNAs, the telomerase complex will assemble properly but that the siRNAs will prevent functional association of telomerase at the telomeres. Using the ChIP assay, if telomerase fails to bind the telomeres, we would expect an inability to precipitate hTERT/telomere complexes. Either MCF-7 cells without siRNA or with the empty vector will serve as negative controls.

Alternative 1: One possible caveat regarding this experiment involves the small amounts of endogenous telomerase at the telomeres, which may pose a detection problem. We can overcome this difficulty by overexpressing an hTERT-TRF2 fusion vector, which we have obtained from Dr. Chris Counter (Duke University, Durham, NC) in the breast tumor cells, which will target significantly more hTERT to the telomeres and has been shown to rapidly elongate telomeres. Because here we expect telomere association, ChIP will be used together with following telomere length. If telomerase is unable to bind at the telomere in the presence of the siRNAs, we will not observe telomere lengthening or a more gradual telomere elongation.

Establishment of the Stable siRNA Knockdown in Breast Tumor Cells: Initially all experiments will utilize the MCF-7 breast tumor cell line but additional lines (MDA-MB241, ZR-75, & T-47D) will also be used to determine if the results represent universal mechanisms. Using the clones that we have already established as mentioned in the preliminary experiments, we will look at the levels of hTR RNA. Expression levels of hTR will be measured using RT-PCR after isolation of total RNA collected throughout colony growth. For hTR-T and hTR-2, the PCR primers are located upstream of the template target and the other downstream of the pseudoknot target so that both will be amplified similarly. If degradation occurs, then we will observe a substantial loss of full-length hTR amplification. Because we find an 80-85% reduction in telomerase activity it is expected that some hTR should be amplified. However, if the hTR is cleaved by RNAi, then a constant level of hTR will be detected, but it will still be

significantly lower than the hTR levels in MCF7 or empty vector control cell lines. Northern Blot analysis will be utilized with total RNA from the cells to not only allow another method to examine and verify levels of hTR expression, but also to assess fragmentation.

Alternative 2: We will utilize an siRNA system developed by Dr. Elizabeth Blackburn's laboratory, where they express hTR siRNA in a lentivral system while co-expressing either exogenous wild-type hTR or 2 template mutant versions (11). These experiments have already been initiated, having determined that the vast majority of the experiments will require transient expression and analysis as stable selection proved fruitless (data not shown). In addition, we are subcloning out the wild-type sequence in order to just obtain the siRNA lentiviral construct. Because there is no antibiotic selection for this, while removing the hTR, we will insert a selectable marker, puromycin or neomycin, in order to allow for selection of stable cell lines. It is quite possible that if this siRNA for hTR is potent, no stable populations will be obtainable. We will utilize these lines to test for sensitization of tumor cells to common therapeutic agents (see Aim #2 below).

Expected Results: The siRNAs targeting hTR have been shown to knockdown telomerase expression. However, the exact mechanism of this phenomenon is not well understood. We hope to clarify the mechanism of the decreased telomerase expression in breast tumor cells. The possibility also exists that because using siRNAs does not cause a total inhibition of expression, there may be more than one pathway for RNAi of telomerase such as partial degradation and partial disruption of RNA folding. The expectation is that all of these mechanisms of hTR degradation or interuption of function will occur to a certain degree and may vary between the cell lines tested.

***Objective #2*: Determine if siRNA knockdown of telomerase sensitizes breast tumor cells to conventional breast cancer therapy.**

Here, we will examine the relationship between telomere length and the DNA damage caused by cancer treatments including Adriamycin (AdR), γ-radiation, and etoposide. In this portion of the study, we are going to attack the cells in two ways: first, by blocking telomerase with the siRNAs, and second, by antisense hTR, both of which will be followed by the addition of one of the three cancer treatments. The first set of cells we will be using will have functional p53 (MCF-7 & ZR-75) and the second set will lack p53 expression (T-47D & MDA-MB231). Wild-type p53 plays an important role in the cellular response to cell damage in that when p53+ cells are treated with AdR, cells undergo replicative senescence; however, cells without p53 undergo a delayed apoptosis after treatment with AdR (5,7).

Assess the effect of the siRNAs individually as a sensitization pretreatment to different types of breast cancer therapy: Our strategy is to suppress telomerase activity using the siRNAs or antisense in an attempt to sensitize the breast tumor cells to therapeutic agents, thus causing a more effective cellular response at a less toxic dose. The four cell lines will be infected by the pSUPER-retro vector with and without the siRNAs, selected with puromycin (0.6μg/μl) and grown to confluency. Then the cells will be split appropriately and exposed for 2

hours to the following therapeutic modalities: AdR (0.1 to 3 μM), etoposide (1-100 μM), and γ-radiation (0.1-10 GY). For all cell lines, there will be at least three replicate plates for each: one for AdR, one for etoposide, and one for γ-radiation. This entire study will be replicated exactly for the each hTR siRNA construct. We will test the breast tumor cells for apoptosis (TUNEL), telomere lengths (TRF & FISH with a telomere specific probe), telomerase activity (TRAP), senescence (SA-βgal), and cytogenetic alterations such as end fusions, telomere breakage or ring chromosomes (karyotyping & SKY).

Alternative 1: Use of more than one siRNA in a single experiment as a double-knock down of hTR doubly may provide optimal sensitization, (i.e. single knockdown may have enough residual activity to prevent significant sensitization). In this situation, we would need to reclone sequences into a vector with an alternative selectable marker (hygromycin or neomycin, which are available) and infect the panel of cell lines to increase telomerase inhibition. We expect to observe a further reduction of telomerase levels than that seen in the single knockdown experiments, which may provide the level of sensitization expected.

Alternative 2: Our laboratory has found that treatment of MCF-7 (p53+) cells induces widespread senescence within the population (more than 99.99%) (5). However, recovery after a single acute dose of AdR consistently occurs and these cells always express telomerase activity (7). Thus, we will look at proliferative recovery in p53 and breast tumor cells to determine if inhibition of telomerase is capable of preventing outgrowth of resistant or recovered cells. This will be done quantitatively by determining the number of recovered clones with and without hTR siRNA at the dose range indicated.

Alternative 3: One possibly important issue in knocking down hTR as a therapeutic target would be its expression in normal cells, as it is ubiquitously expressed. This alternative would involved the use of normal or preimmortal mammary epithelial cells (6), which express hTR but not hTERT, as a means to determine the sensitization of these cells to similar therapeutic modalities as above.

Expected Results: The decrease in telomerase levels and telomere length caused by the pre-treatment with the hTR siRNAs (or antisense) will ultimately make the telomeres more vulnerable to the AdR, etoposide and γ-radiation. This will result in higher levels of telomere dysfunction and chromosomal abnormalities due to the lack of telomeric repeats to protect the chromosome ends. The increased susceptibility of the cells to DNA damage will be critically important in the induction of apoptosis

***Objective #3*: Determine if siRNA knockdown of p21 sensitizes breast tumor cells to conventional breast cancer therapy.**

Our lab has previously shown the molecular and cellular consequences of Adriamycin treatment in breast tumor cells (5,7). After acute exposure to Adriamycin, MCF-7 cells senescence approximately three days later and down-regulate telomerase. Telomere length has been proven to be an important trigger for senescence. However, we have shown that senescence can be induced

without net shortening of the telomeres (5). That the most critical event is the preservation of telomere structure/integrity and p53 levels only when this occurs do we have AdR-induced senescence in MCF-7 cells. Adriamycin and ROS have been shown to preferentially attack the telomeres (5). Senescence is also characterized by transient p53 activation, high levels of reactive oxygen species as well as sustained $p21^{waf-1}$ expression. From these results I wanted to further examine and understand the relationship between p21 and senescence. Perhaps there is a threshold of p21 needed to maintain the senescence phenotype, and if I can knock down the p21 protein levels enough then the cell should undergo apoptosis instead of senescence.

Assess the effect of the p21 knockdown via RNAi as a sensitization pretreatment to different types of breast cancer therapy: We obtained the siRNA p21 sequence from Bernards et al. (1), which targets the third exon very shortly after the start codon, and stably expressed the siRNA in the MCF-7 cells (Figure 5). The pSUPER-retro vector was utilized again, which functions in the same manner as mentioned before, except this retroviral vector has a GFP marker so that positive clones can be selected visually and via antibiotic resistance. Once infected into the cell lines and selected, the clones were screened for constitutive levels of p21 by Western Blotting (Figure 5). There were varying levels of knockdown with the highest percentage ranging around 80%.

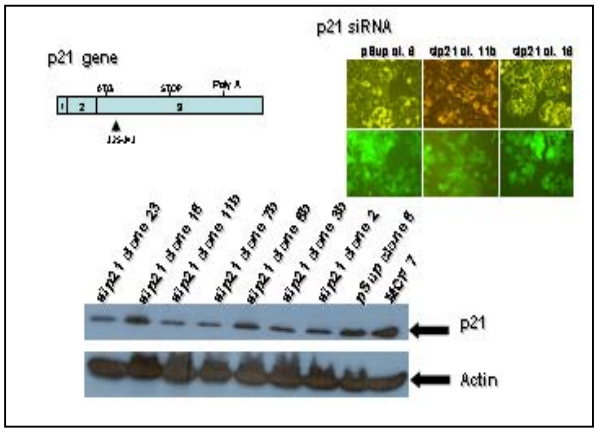


Figure 5. Knockdown of functional p21 in MCF7 cells by siRNA. MCF7 breast cancer cells were stably infected with siRNA directed at p21. The clones were visualized and selected upon expression of GFP. Protein levels of p21 were decreased ~80% in several of the clones. However, there was a range of reduction showing differences in infection efficiencies.

Following selection of three of the clones with different constitutive amounts of p21, we administered acute Adriamycin treatment (1μM) to the MCF-7/p21 cell lines for 2 hours. Protein levels of p21 were determined at 0, 4, 24, and 48 hours post treatment using Western Blot (Figure 6). After four hours induction of the p21 occurs and continues to be elevated up to 48 hours. To check these results we ascertained protein levels 24 hours after AdR treatment using immunohistochemistry. Again we saw decreased constitutive amounts of p21 and then generation of high levels after treatment (Figure 6).

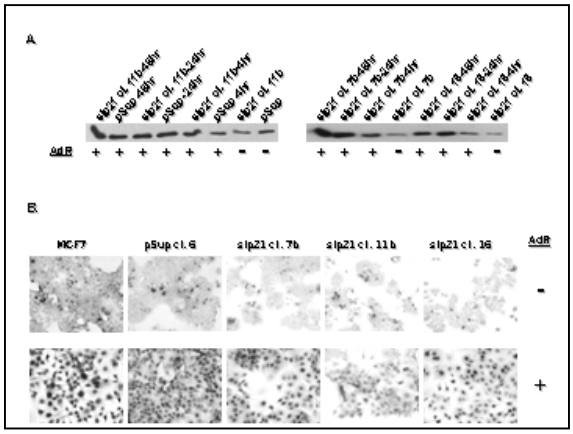


Figure 6. p21 induction after AdR treatment in MCF7/p21 knockdown clones. Using the Western Blot (A) and immunohistochemistry (B), p21 levels were measured at various time points before and after treatment. In both experiments the siRNAs were shown incapable of continued suppression of p21 post treatment with adriamycin.

We also looked at senescence after AdR treatment and found increases in the rate of senescence. The same three clones with decreased levels of p21 were treated for 2 hours with Adriamycin (1μM). Untreated MCF-7 cells undergo senescence three days after treatment; whereas, in the MCF/p21 cell lines with the greatest amount of knockdown, senescence was apparent 24 hours after treatment and continued for at least five days afterwards (Figure 7). However, clone 16, with the most normal amount of p21, and the empty vector control displayed similar amounts of SA-βgal staining by day 3 (Figure 7). This experiment needs to be repeated and quantitated before more conclusions can be made.

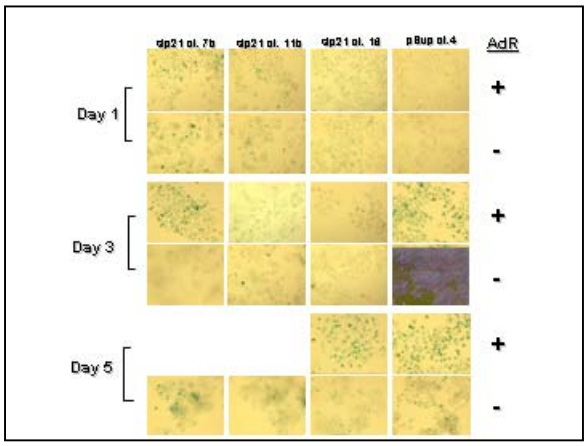
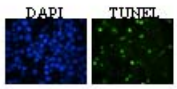


Figure 7. Senescence initiation in MCF7/p21 knockdown clones. By comparing the quantities of SA-βgal staining in the three p21 knockdown clones to the empty vector (pSup) clone, we determined the senescent phenotype occurs at a faster rate in the clones with lower levels of p21 after AdR treatment.

The cell lines were also tested for apoptosis (TUNEL) after acute Adriamycin treatment. We found increases in the amount of cell death as compared with the empty vector control cell lines (Figure 8). This portion of the project is not complete, and the data presented is quite preliminary and needs to be repeated due to varying results collected in the analysis, as well as increasing the number of time points analyzed.

A.



Clone 11b-DAY 9 (26%)

B.

Sample	Clone 7b	Clone 11b	Clone 16	pSup cl. 6
UNTREATED				
Day 5	0	.2%	.65%	0
Day 7	0	0	.45%	0
TREATED				
Day 7	9.7 % (n=2)	9.3 % (n=3)	5.9 % (n=3)	1.7 % (n=3)
Day 9	20.7 % (n=6)	22.1 % (n=6)	21.8 % (n=6)	

Figure 8. Apoptosis in MCF7/p21 knockdown clones after acute AdR treatment. The TUNEL assay enables a comparison of the DAPI staining of the nuclei of each cell and the fluorescently labeled dead cells but TUNEL (A). From the samples taken, there appears to be significantly greater amounts of apoptosis occurring in the MCF-7 cells lines with p21 knockdown regardless of the level of decrease in protein.

We have clearly shown that blocking p21 function results in a transient decrease after treatment with Adriamycin followed by a large amplification in protein quantities. This constitutive 80% knockdown of p21 levels does not appear to be significant enough to be maintained post treatment with AdR. We observed the majority of the cells senescencing while others appear to undergo apoptosis. The reason for this result is still unknown and is under investigation. Because we see p21 knockdown with siRNA before but not after DNA damage, we will explore the option of homologously knocking out p21 in the MCF-7 cell line as a cleaner system for p21 knockout.

Key Research Accomplishments

1-athough transient inhibition of telomerase activity was observed, using RNAi to knockdown levels of hTR was not as effective as expected. A significant stable decrease in telomerase activity was not observed *in vivo*.

2-*in vitro* studies of siRNAs and hTR have shown that the synthetic siRNAs do not interfere with the degradation or secondary structure of functional hTR.

3-levels of p21 were decreased by ~80% using RNAi in breast tumor cell lines.

4-acute adriamycin treatment of MCF-7/p21 knockdown cell lines causes an induction of p21, and the RNAi occurring in these cell lines does not suppress DNA damage-induced activation of p21 after treatment.

Recommended Changes to the Proposed Work Based on Additional Findings

In order to complete the studies in Aim #1 related to *in vitro* and *in vivo* study of RNA interference, we will be utilizing the pSuper.retro/GFP vector for the rest of the hTR studies in breast tumor cell lines. We will also add the expression of using the lentiviral system developed by Dr. Elizabeth Blackburn's ground (11), and eliminate the wild-type sequence from the vector, as well as test for sensitization with the dual expression of hTR siRNA and mutated hTR sequences. In addition, a more thorough study of p21 and senescence will be conducted. No additional changes to the proposal are requested.

Abstracts/Presentations

Poynter, K.R., L.W.Elmore, and S.E.Holt. Era of Hope: Department of Defense Breast Cancer Research Program Meeting. Philadelphia, PA. June 2005.

Development of Cell Lines
We have developed cell lines for telomerase knockdown with two different targets on hTR and a knockdown of p21 in MCF-7 cells. We have also obtained lentiviral vectors for knockdown of hTR with siRNA from Dr. Elizabeth Blackburn (UCSF, San Francisco, CA) and are creating stable MCF-7 cell lines (11). Other siRNA vectors that have been constructed and expressed in breast tumor cells but not completely characterized include several MCF-7 cell lines that have targeted knockdown of hTERT, using RNA interference (4 different siRNA targets) and using a dominant-negative approach to blocking telomerase (DN-hTERT).

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Conclusions

Having explored several avenues of RNA interference, *in vitro* using synthetic siRNAs and *in vivo* using retroviral infection of siRNAs, we are clearly on pace to determine what occurs when directing this mechanism at a functional RNA. Using RNAi to knock-down levels of hTR were not as effective as expected. The significant decreases in telomerase activity that were expected were not observed *in vivo*. We have also utilized siRNAs to attack p21 in order to further examine this cellular signaling protein's role in senescence and apoptosis. Our data has shown that even with a high percentage knockdown, this protein is capable of returning to greater than normal levels after treatment with adriamycin for two hours. The resulting phenotype in these cell lines from AdR treatment lying somewhere in between senescence and apotosis. Inhibition of telomerase and p21 in breast tumor cells in combination or alone may provide a novel mode of cancer therapy and will be critically important for the rationale design of new adjuvant therapies for breast cancer patients.

Abbreviations

hTERT-human telomerase reverse transcriptase; **hTR**-human telomerase template RNA; **AdR**-adriamycin; **siRNA**-short interfering RNA; **RNAi**-RNA interference; **SKY**-spectral karyotyping; **FISH**-fluorescent *in situ* hybridization; **SA-β gal**-senescence associated β -galactosidase; **ChIP**-chromatin immunoprecipitation; **TRAP**- telomere repeat amplification protocol (telomerase activity assay); **TRF**- terminal repeat fragment (telomere length assay); **TRF2**- telomere repeat binding factor number 2; **IP**- immunoprecipitation; **TnT**- transcription and translation.

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